

## A Real Time PCR strategy for the detection and quantification of *Candida albicans* in human blood

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### ABSTRACT

Candidemia is a significant cause of bloodstream infections (BSI) in nosocomial settings. The identification of species can potentially improve the quality of care and decrease human mortality. Quantitative PCR (qPCR) was evaluated for *Candida albicans* detection using culture suspensions containing *C. albicans*, spiked human blood, the cloned qPCR target fragment (ITS2 region) and the results of these assays were compared. The assays showed a good detection limit: *C. albicans* DNA extracted from yeast (sensitivity 0.2 CFU/μL), spiked human blood (sensitivity 10 CFU/mL), and cloned fragment of ITS2 region (sensitivity 20 target copies/μL). The efficiency of ITS2 fragment-qPCR ranged from 89.67 to 97.07, and the linearity ( $R^2$ ) of the standard curve ranged from 0.992 to 0.999. The results showed that this ITS2-qPCR has a great potential as a molecular prototype model for the development of a test to be applied in clinical practice, greatly reducing the time of candidemia diagnosis, which is extremely important in this clinical setting.

**KEYWORDS:** *Candida albicans*. Candidemia. Detection. Invasive candidiasis. ITS2. Quantitative PCR.

### INTRODUCTION

Candidemia is the fourth most common cause of bloodstream infections (BSI) in nosocomial settings, with up to 50% of episodes occurring in intensive care units (ICU)<sup>1</sup>. Patients with invasive candidiasis (IC), even receiving antifungal therapy, have a mortality rate of 40%<sup>2</sup>. Furthermore, candidemia increases the hospital length of stay and the total cost of medical care<sup>3</sup>. Girão *et al.*<sup>4</sup> from Universidade de São Paulo (USP), evaluated nosocomial candidemia in an ICU at Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP) for seven years, and they assessed 8.3 to 10.8 cases of BSI of 1,000 patients per day, and 5.6% of the cases were caused by *Candida sp.* The authors observed that *C. albicans* caused the majority of infections (40%), followed by *C. tropicalis* (34%), *C. parapsilosis* (18%), *C. krusei* (3%), and *C. guilliermondii* (1%)<sup>4</sup>.

The diagnosis of *Candida sp.* BSI is challenging, and, in general, takes place in the later stages of the disease. The blood culture is currently the gold standard for the diagnosis of IC; however, it has low sensitivity, and takes 48-96 h to identify the species<sup>5</sup>. The need of new tools for the diagnosis of candidemia is important due to the low sensitivity of blood culture, as only about 50% of all cases of candidemia are

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detected<sup>6</sup>. Non-culture methods could successfully increase the sensitivity of the early diagnosis of IC<sup>7</sup>.

Quantitative PCR (qPCR) is considered a sensitive and accurate technique. The main advantage of this technique is the ability to generate quantitative data, in contrast to qualitative data generated by conventional PCR. Among the most promising targets for the diagnosis of invasive fungal infections by qPCR, the ITS (Internal Transcribed Spacer) region is part of the ribosomal RNA (rDNA) gene complex and has been used as a target in candidemia diagnosis by PCR. It is a non-coding region, and the genomic sequences are present in around 55 copies per haploid genome<sup>8</sup>.

The aim of this study is to evaluate an in-house qPCR specifically directed to *C. albicans*, applying a standard curve of a cloned fragment of the ITS2 region as a reference in future qPCR assays for the detection and quantification of *C. albicans* in human blood. This new assay should have a good detection limit, reproducibility and specificity.

## METHODS

This study was approved by Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de São Paulo (Process N° 1.856.53). Blood samples from healthy participants were collected after the signature of an informed consent. The samples were stored at 4 °C until processing.

Yeast cells of *C. albicans* (ATCC 90028) were cultivated in Sabouraud agar plates (37 °C; 24 h). They were added in a solution of Tris-HCl-EDTA (TE) and counted in a Neubauer chamber; subsequently, they were adjusted to a concentration of 10<sup>8</sup> colony forming units per mL (CFU/mL).

Three sets of experiments were performed:

### 1) *C. albicans* suspension in a concentration of 10<sup>8</sup> CFU/mL

For DNA extraction, the pellet containing the yeasts was incubated at 37 °C for two h in TE (Tris HCl 10 mM pH 8.0; EDTA 2 mM pH 8.0) and 200 U of Lyticase (Sigma-Aldrich, Missouri, USA). The remaining steps were performed by using the QIAamp® DNA Blood Mini Kit (QIAGEN Inc., Hilden, Germany), following the manufacturer's instructions. The sample was eluted in 100 µL of an elution buffer (QIAGEN). DNA concentration was estimated by using a UV spectrophotometer (Nanodrop LITE, Thermo Fisher Scientific Corp., Wilmington, Delaware, USA). A ten-fold dilution series, from 10<sup>6</sup> to 10<sup>-3</sup> CFU/µL of the product of extraction, were submitted to amplification.

### 2) Spiked blood samples with *C. albicans*

*C. albicans* yeasts were added to non-infected human

blood in a concentration of 10<sup>7</sup> CFU/mL, and immediately homogenized. The spiked blood was then used to produce a ten-fold dilution series (10<sup>7</sup> to 10<sup>0</sup> CFU/mL). To other two blood aliquots of 1 mL each, 2 and 5 yeasts were added. An extraction control and blood without yeasts were processed along with the spiked curve dilutions

DNA extraction was performed by following the methodologies described previously<sup>9</sup>, with some modifications: each 1 mL of the spiked blood samples was initially treated with the blood lysis buffer (NH<sub>4</sub>Cl 155 mM; KHCO<sub>3</sub> 10 mM; EDTA 1 mM pH 7.4), followed by a centrifugation at 1,200 g for 15 min; the pellet was incubated at 37 °C for 2 h with Nucleo Lysis Buffer (Tris HCl 10 mM pH 8.0; NaCl 0.4 mM; EDTA 2 mM pH 8.2), and 200 U of Lyticase (Sigma-Aldrich, Missouri, USA) were added. The remaining steps were performed by using the QIAamp® DNA Blood Mini Kit, following the manufacturer's instructions.

### 3) Cloning the fragment of the ITS2 region

A 108-bp DNA fragment of ITS2 region was amplified from a *C. albicans* strain (Accession N° KT876203.1), using the primers forward 5'-GGGTTTGCTTGAAAGACGGTA-3' and reverse 5'-TTGAAGATATACGTGGTGGACGTTA-3' in the qPCR<sup>10</sup>. Then, the PCR product was cloned into the plasmid vector pJET 1.2/blunt, using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific Corp., Wilmington, Delaware, USA), following the manufacturer's instructions. The pJET 1.2/blunt of the ITS2 region linked to the plasmid was purified using the miniprep technique<sup>11</sup>. The DNA concentration was estimated by using a UV spectrophotometer (Nanodrop LITE, Thermo Fisher Scientific, Wilmington, Delaware, USA). In order to generate standard curves for the quantitative assays and to assess the amplification efficiency, a ten-fold dilution series of the pJET 1.2/blunt of the ITS2 region in the plasmid were prepared in 10 mM Tris-EDTA buffer (2 x 10<sup>9</sup> to 2 x 10<sup>0</sup> copies/µL) and stored at -80 °C until use. To minimize contamination, the preparation of the pJET 1.2/blunt of the ITS2 region plasmid dilutions was carried out in a separate room of the laboratory.

The lowest concentration of *C. albicans* detected by qPCR was the detection limit, which was estimated by the dilution series, tested in three sets of experiments.

The qPCR assays were performed in 20 µL of total volume containing 10 µL of 2 X Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, California, USA), 0.2 mM primers (region ITS-2) and 5 µL of each DNA sample. A StepOne Plus Real Time PCR System (Applied Biosystems, Carlsbad, California, USA) was used. The cycling conditions consisted of an initial denaturation

step at 95 °C (5 min), followed by 40 cycles at 95 °C (30 s), 58 °C (30 s), and 72 °C (30 s), and a final dissociation step at 56-95 °C with a heating rate of 0.3°C/s.<sup>10</sup> To estimate the equivalence between CFU and femtograms (fg) a previously reported methodology was followed<sup>12</sup>.

The assay reproducibility in detecting the cloned ITS2 fragment and spiked blood samples was evaluated in 15 independent assays: the first included ten assays with ten-fold dilution series of the cloned fragment ( $2 \times 10^9$  to  $10^0$  copies/ $\mu$ L) and the second 5 assays with ten-fold dilution series of spiked blood ( $10^7$  to  $10^0$  CFU/mL). Both assays were performed in different days under the same conditions.

The qPCR specificity was evaluated by testing DNA samples from other *Candida* species, other fungi and bacteria (*C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. dubliniensis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*; *Rhodotorula sp*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Fusarium sp*) in a DNA concentration of 5 ng/reaction. As the positive control for these reactions, *C. albicans* DNA was used at concentrations of 0.05 and 5 ng/reaction.

In addition, two negative controls (No Template Control - NTC) were included in each assay: one reagent control and one control to investigate contamination during the application of the standard curve samples.

The SPSS Statistics 17.0 software (SPSS Inc., Chicago, Illinois, USA) was used to determine the mean values and the standard deviations based on independent experiments for each set.

## RESULTS AND DISCUSSION

For each of the two standard curves, five independent experiments were performed from a dilution series of

spiked human blood, and ten independent experiments were performed from the cloned fragment of *C. albicans*, respectively.

Standard TS2 curves generated by ten-fold dilutions series of DNA from  $2 \times 10^9$  to  $2 \times 10^0$  copies/ $\mu$ L concentrations showed a perfect agreement in all ten independent qPCR assays. The assays demonstrated high efficiency (between 89.03% to 101.46%), and the other critical parameter evaluated, the linearity  $R^2$  was between  $0.996 \pm 0.003$  (Table 1). The detection limit of the cloned ITS2 target indicated a mean Ct value of  $32.97 \pm 0.99$ , corresponding to 20 copies/ $\mu$ L (Table 2 and Figure 1A). These results are similar to the other experiment in which the detection limit was 80 copies/mL in serum or plasma samples from IC patients comparing the samples to a standard curve generated by diluted plasmids from  $10^8$  to  $10^1$  copies/reaction<sup>13</sup>.

The detection limit observed in qPCR assays with DNA samples of *C. albicans* from culture was  $10^{-1}$  CFU/ $\mu$ L, which corresponds approximately to 5 fg/reaction (Table 2 and Figure 1B). This value is compatible with the results found in the limit dilution of the cloned sequence, i.e. 20 copies/reaction, which corresponds approximately to one-fifth of a single CFU, considering approximately 110 copies of the sequence/CFU (diploid genome)<sup>8</sup>. Other authors who used *C. albicans* culture samples in qPCR observed similar results<sup>14,15</sup>.

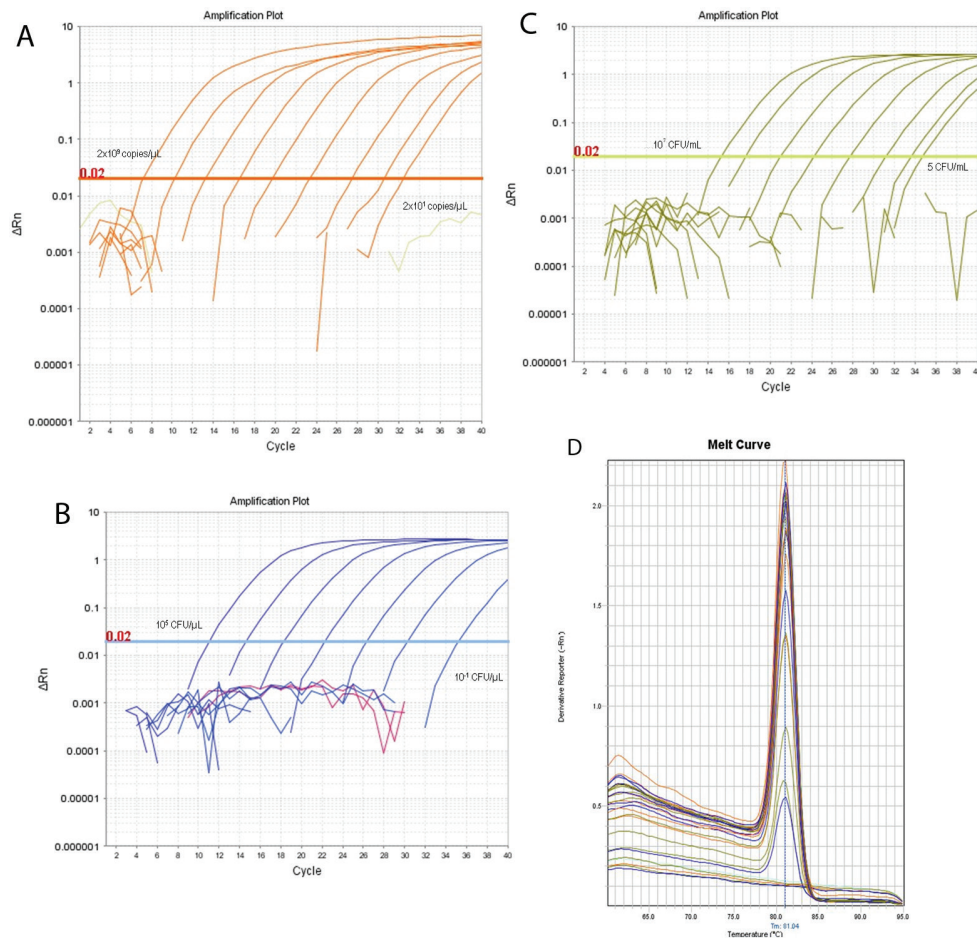
**Table 1** - qPCR performance to detect *Candida albicans* using cloned target sequence and spiked blood

	Cloned target	Spiked blood
Number of replicates	10	5
Slope (mean $\pm$ SD)	-3.452 $\pm$ 0.115	-3.515 $\pm$ 0.226
Linearity ( $R^2$ )	0.996 $\pm$ 0.003	0.997 $\pm$ 0.002
Efficiency (%)	95.184 $\pm$ 4.237	97.056 $\pm$ 4.088

**Table 2** - Performance of qPCR in three sets of experiments

Cloned Target*		<i>C. albicans</i> culture**		Spiked Blood**	
Copies/ $\mu$ L	Ct Mean $\pm$ SD	CFU/ $\mu$ L	Ct Mean $\pm$ SD	CFU/mL of Blood	Ct Mean $\pm$ SD
$2 \times 10^{-1}$	ND			$2 \times 10^0$	ND
$2 \times 10^0$	ND		ND	$5 \times 10^{0***}$	ND
$2 \times 10^1$	32.98 $\pm$ 1.00	$10^{-1}$	33.70 $\pm$ 1.53	$10^1$	33.01 $\pm$ 0.65
$2 \times 10^2$	29.54 $\pm$ 0.85	$10^0$	30.94 $\pm$ 0.73	$10^2$	34.70 $\pm$ 0.69
$2 \times 10^3$	26.16 $\pm$ 0.86	$10^1$	27.13 $\pm$ 0.49	$10^3$	30.36 $\pm$ 1.55
$2 \times 10^4$	22.40 $\pm$ 0.73	$10^2$	22.66 $\pm$ 0.68	$10^4$	26.33 $\pm$ 1.12
$2 \times 10^5$	18.89 $\pm$ 0.92	$10^3$	18.35 $\pm$ 0.52	$10^5$	23.80 $\pm$ 0.81
$2 \times 10^6$	15.38 $\pm$ 0.82	$10^4$	14.80 $\pm$ 0.48	$10^6$	20.32 $\pm$ 0.74
$2 \times 10^7$	11.89 $\pm$ 0.77	$10^5$	11.02 $\pm$ 0.30	$10^7$	17.54 $\pm$ 0.84

\* mean of ten assays; \*\* mean of three assays; \*\*\* mean of five assays; ND - non detected



**Figure 1** - Technical performance and detection range of the *Candida albicans* qPCR: A) Amplification curves of ten-fold dilutions of cloned targets ranging from  $2 \times 10^9$  to  $2 \times 10^1$  copies/ $\mu$ L; B) Amplification curves of ten-fold dilutions of cultures, ranging from  $10^5$  to  $10^{-1}$  CFU/ $\mu$ L; C) Amplification curves of ten-fold dilutions of spiked blood, ranging from  $10^7$  to  $10^1$  and 5 CFU/mL of blood; D) Dissociation curves for the amplifications observed in A, B and C, where the same Melting temperature of  $81.06 \pm 0.19$  °C was observed. The linearity  $R^2$  value was 0.996.

The present study allowed the detection of 10 CFU/mL of spiked blood samples in 100% of experiments and a detection of 5 CFU/mL in 50% of the same experiments. (Table 2 and Figure 1C). In the spiked blood samples, using similar methods, other researchers found 1 CFU/mL in 25%, 10 CFU/mL in 83%, and 100 CFU/mL in 100% of the experiments<sup>16</sup>. Previous studies have also shown the limit of detection of 10 CFU/mL of blood<sup>17-19</sup>. One of them compared extraction methods as well, observing that the QIAamp® DNA Blood mini kit (QIAGEN) was more sensitive than the other methods in serum samples spiked with *C. albicans*<sup>17</sup>. The qPCR assays with spiked blood were as efficient as the assays using the cloned target sequence, with the efficiency ranging from 89.67 to 97.07, and the linearity  $R^2$  from 0.992 to 0.999 (Table 1).

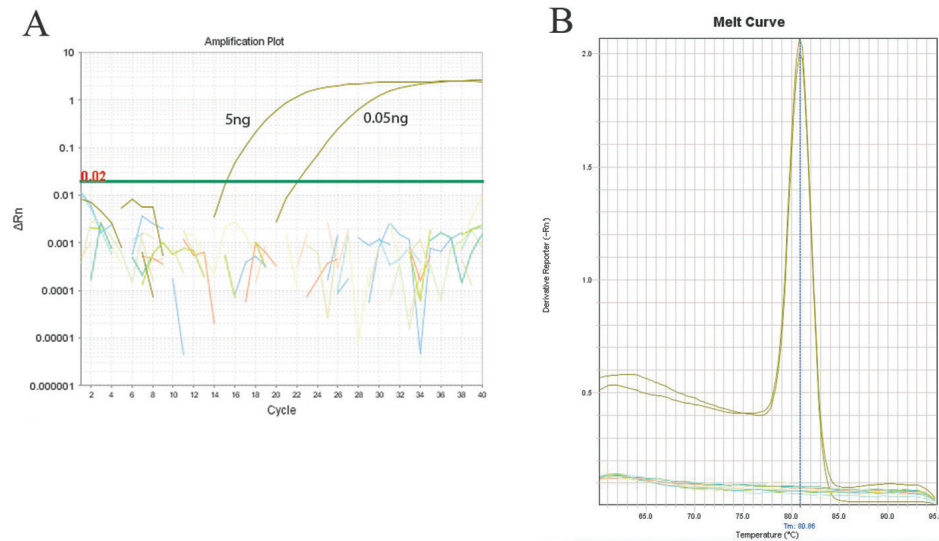
It is important to highlight that in all the assays carried out with cultured *Candida albicans*, cloned qPCR target sequence and spiked blood, the dissociation curves (melt curve) revealed the same temperature of  $81.06 \pm 0.19$  °C

(Figure 1D). The qPCR had also a high analytical specificity as all other DNA samples from other fungal and bacterial strains tested negative (Figure 2). Similarly, a study using the TaqMan system and the same primers used in the present study did not find cross reactions with the 13 species of *Candida* tested, or with more than 20 tested species of other fungi and bacteria<sup>10</sup>.

One of the advantages of qPCR is the possibility of an earlier diagnosis (positive qPCR results were reported in patients who subsequently developed candidemia or clinical signs of IC). Moreover, the possibility of monitoring the persistence or resolution of the infection<sup>7</sup> is also interesting for the patients' follow-up. A high sensitivity of qPCR was demonstrated when it was compared to blood cultures in patients with proven/probable or possible IC<sup>7</sup>.

In a retrospective study, the authors have shown the ability of qPCR in detecting less than 5 CFU/mL in 23% of cultured peripheral blood samples from patients with known IC<sup>6</sup>, and the time required by the molecular technique for





**Figure 2** - Specificity: A) qPCR results with 5 and 0.05 ng of *C. albicans*. DNA samples from various microorganisms were tested: *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. dubliniensis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Rhodotorula sp.*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Fusarium sp.*; B) Dissociation curves for the amplifications observed in A (melting temperature of 80.86 °C).

diagnosis is still an advantage when comparing it with cultures, since blood cultures depend on the growing time of yeasts.

Studies with clinical samples have shown that only 53% of positive cultures had fungal density lower than 1 CFU/mL, in the first culture-positive sample<sup>20</sup>, however, it is necessary to establish a relationship between the amount of CFUs present in the blood sample and the amount of CFUs that have grown in the culture plate. This correlation was demonstrated in a previous study involving experiments with spiked blood, and the authors pointed to a detection limit for *C. albicans* of 10<sup>3</sup> CFU/mL for aerobic and 10<sup>1</sup> to 10<sup>2</sup> CFU/mL for anaerobic blood cultures<sup>21</sup>, which could explain the low sensitivity of the culture method in the detection of *Candida sp.* in blood samples.

Further studies are still necessary to improve and implement the qPCR technique, evaluating its impact on clinical practice. This study has shown that throughout the analyzed parameters it was possible to construct standard qPCR curves with cloned target sequences, reaching a good limit of detection, reproducibility and great specificity. The method used is a prototype strategy based on molecular technology for the development of future assays focused on the detection of *C. albicans* and other *Candida sp* in human blood to accelerate the identification of the yeast, allowing a rapid and more appropriate clinical intervention.

## AUTHORS' CONTRIBUTIONS

Felipe Delatorre Busser and Vera Lucia Teixeira de Freitas conceptualized, designed the experiments; Felipe

Delatorre Busser, Vivian Caso Coelho and Claudia de Abreu Fonseca carried out molecular assays and the initial analyses; Marta Heloisa Lopes, Gilda Maria Barbaro Del Negro, Marcello Mihailenko Chaves Magri and Maria Aparecida Shikanai-Yasuda contributed to the writing and they critically reviewed the manuscript; Felipe Delatorre Busser, Vivian Caso Coelho and Vera Lucia. Teixeira de Freitas drafted the initial manuscript and reviewed the manuscript. All the authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

## CONFLICT OF INTERESTS

The authors have no conflicts of interest to disclose.

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## FINANCIAL DISCLOSURE

The authors have no financial relationships relevant to this article to disclose.

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